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Phylogenetic Affiliation of BEV, a Bacterial Parasite of the Leafhopper *Euscelidius variegatus*, on the Basis of 16S rDNA Sequences

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Abstract. The phylogenetic relationship of a nonflagellated, Gram-negative, rod-shaped intracellular bacterial parasite (BEV) of the leafhopper *Euscelidius variegatus* to other bacteria within the class *Proteobacteria* was determined by sequence analysis of 16S rDNAs. The presence of specific signature nucleotides showed this bacterium to be a member of the γ-3 subdivision of the *Proteobacteria*. Phylogenetic analysis based on maximum parsimony placed BEV within a clade in the *Enterobacteriaceae,* which includes a number of bacteria that are facultative symbiotes of insects and have a common ancestor with *Proteus vulgaris*. Within this clade, BEV is most closely related to a bacterium identified as the secondary endosymbiote of another homopteran, the pea aphid, *Acyrthosiphon pisum.*

Many homopterous insects (e.g., aphids, scale insects, whiteflies, leafhoppers, etc.) have an intimate association with bacteria that are harbored intracellularly. Because of their physiological and biochemical dependence on the host, these bacteria either grow poorly in culture or are unculturable outside of the host insect. In the past, the difficulty in growing these bacteria has prevented the determination of their taxanomic affiliation [3, 10, 24, 25]. This problem can now be circumvented by using 16S rRNAs or their genes (16S rDNAs) as a basis for estimating the phylogenetic affiliation of bacteria [27]. Recently, the phylogenetic positions of endosymbiotic bacteria of a number of homopterans in the suborder Sternorrhyncha have been determined with this approach (aphids [15], mealybugs [16], and whiteflies [5]). To date, none of the intracellular bacteria associated with insects in the other suborder of the Homoptera, Auchenorrhyncha (leafhoppers, planthoppers, etc.) has been identified [10, 24].

A Gram-negative, rod-shaped bacterium was recently found to infect various internal organs of adults and nymphs and eggs of a leafhopper, *Euscelidius variegatus*. This leafhopper is commonly found on a wide variety of plants throughout Europe, Asia, and North America [9]. This bacterium, given the trivial designation of BEV, was found in all individuals examined in colonies of *E. variegatus* in France, but was not present in individuals from laboratory colonies in California. Uninfected females of *E. variegatus* inoculated with cultures of BEV transmitted the bacteria to their progeny. The infected offspring had significantly reduced longevity and fecundity and required a longer period for nymphal development. However, BEV was highly pathogenic when injected into other species of leafhoppers. On the basis of these observations, BEV has been regarded as a facultative bacterial parasite of *E. variegatus* [18, 19].

Euscelidius variegatus is a vector of many plant pathogenic mollicutes including *Spiroplasma citri* [13], and etiological agents of clover phyllody [8], aster yellows [22], and X-disease [11]. Current interest in BEV results from the observation that individuals of *E. variegatus* infected with BEV show a significantly reduced ability to transmit several plant pathogens. BEV is also a lethal pathogen to several other species of leafhoppers. Attempts to determine the definitive taxonomic identity of BEV by use of morphological and physiological characteristics have been unsuccessful [19]. In this report, we present the phylogenetic position of BEV within the

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Proteobacteria on the basis of molecular phylogenetic analysis of 16S rDNAs.

Materials and Methods

Bacteria were isolated from a colony of *E. variegatus* that had been infected years earlier by injection of nymphs with a suspension of BEV [19]. The medium used for isolation was Difco purple broth with 1.5–2.0% agar, acidified to pH 6.3 with 0.1 *N* HCl. Lawns of cultured stocks were prepared on purple agar plates covered with aluminum foil and incubated at 37°C overnight. The procedures used for determining morphology, physiology, and biochemical characteristics were described previously [19].

Procedures for isolation, amplification by polymerase chain reaction (PCR), and phylogenetic analysis of 16S rDNAs were similar to those previously described [4]. The following is a summary of these procedures. Genomic DNA was isolated from bacteria harvested from plates according to the methods of Sambrook *et al.* [21]. PCR [20] was used to selectively amplify double-stranded 16S rDNA for 30 cycles according to procedures outlined in the GeneAmp® kit (Perkin Elmer/Cetus, Norwalk, Connecticut). The PCR primers were: forward 5′-CAT GGC TCA GAT TGA ACG CGT GCG-3′, and reverse 5′-CCC CTA CGG TTA CCT TGT TAC GAC-3′ (positions 18–41 and 1494–1517 of the *E. coli* numbering system, respectively, [1]). Cloning of the amplified 16S rDNA was performed with the TA Cloning™ ver. 1.0 kit (Invitrogen, San Diego, California). Both strands of 16S rDNA clones were sequenced with the Sequenase® ver. 2.0 DNA sequencing kit (US Biochemical, Cleveland, Ohio).

Preliminary analysis of the sequence similarity of BEV 16S rDNA to other eubacterial 16S rDNAs was performed by a *ktup* algorithm search [12] of 16S rDNA sequences deposited with GenBank® (Release 71.0, March 15, 1992) with the GeneWorks ver 2.0 computer program (Intelligenetics, Mountainview, California). The phylogenetic affiliation of BEV was determined by parsimony analysis of 16S rDNA sequences using PAUP [23]. Candidate taxa used for parsimony analysis included bacteria which showed similarity to BEV, as indicated by the database search, and other bacteria known to have an intracellular association with arthropods. All these bacteria were in the γ-3 subdivision of the *Proteobacteria*. Sequences were aligned according to conserved regions of sequence and secondary structure [17]. The tree was rooted with *Wolbachia persica,* a tick-borne bacterium more closely affiliated with bacteria in the γ-2 than the γ-3 subdivision [5, 26]. Transitions and transversions were weighted equally, gaps were scored as missing data, and uninformative sites were ignored. The data matrix consisted of 300 informative characters and 12 taxa. The parsimony analysis was performed by use of the "bootstrapping" option to obtain a 50% majority-rule consensus tree after 100 replications.

Results and Discussion

PCR amplification, cloning, and sequencing. Amplification of 16S rDNA of BEV yielded a singular band of approximately 1500 bp. Three clones with the full 16S rDNA insert were obtained (pBEV-2, 4, and 9). All three clones yielded identical fragment patterns with respect to digestion with either *Eco*RI, *Alu*I or *Hph*I endonucleases. The 16S rDNA of BEV had a GC composition of 54% and possessed particular signature sequences representative of the γ-3 subdivision [28]. The sequence is presented in Fig. 1 and is also deposited with EMBL as accession number Z14096 BEV16SRRN.

Phylogenetic analysis of BEV 16S rDNA. The phylogenetic analysis of 16S rDNAs placed BEV in a monophyletic clade which included a number of bacteria in the *Enterobacteriaceae* (Fig. 2). The formation of this clade was represented by a relatively high bootstrap confidence index of 95. This clade included a number of other bacteria considered to be symbiotes of insects. These symbiotic bacteria included the "son-killer", *Arsenophonus nasoniae,* which prevents development of unfertilized eggs (male offspring) in a parasitic wasp [7]; the bacterial symbiotes of rhynchophorine weevils in the genus *Sitophilus* [4], and the secondary symbiote of the pea aphid, *Acyrthosiphon pisum* [25]. This clade of insect bacterial symbiotes also included the well-known enteric, *Proteus vulgaris. Escherichia coli* was placed in a branch outside of this clade. The primary aphid endosymbionts, *Buchnera aphidicola* [14], were firmly placed in a clade paraphyletic to the *Enterobacteriaceae* as indicated by a bootstrap index of 100. The inclusion of aphid primary endosymbionts in a monophyletic group of the γ-3 subdivision outside the *Enterobacteriaceae* in our analysis is in accordance with previous findings [15]. *Ruminobacter amylophilus* was placed ancestrally to the other members of the γ-3 subdivision analyzed, in agreement with earlier findings [15].

Conclusions

1. Of the bacterial taxa examined in our analysis, BEV has the closest evolutionary relationship to a bacterium in the *Enterobacteriaceae* referred to as the secondary symbiote of the pea aphid. This aphid is the only aphid in which this bacterium has been detected (unlike *B. aphidicola,* which has been found in all aphids examined to date [15]).

2. This finding is of interest in view of the fact that the pea aphid and *E. variegatus* are both members of the insect order Homoptera, and both bacteria are presumably restricted in their host-species range. These two homopteran symbiotes are probably distinct species, as indicated by only 88% homology of nucleotides in their 16S rDNAs [6] (Fig. 1).

3. Future efforts to determine the phylogenetic affiliation of the symbiotic bacteria of other leafhoppers and related homopterans [2, 10, 24] should reveal whether these bacteria constitute a coherent

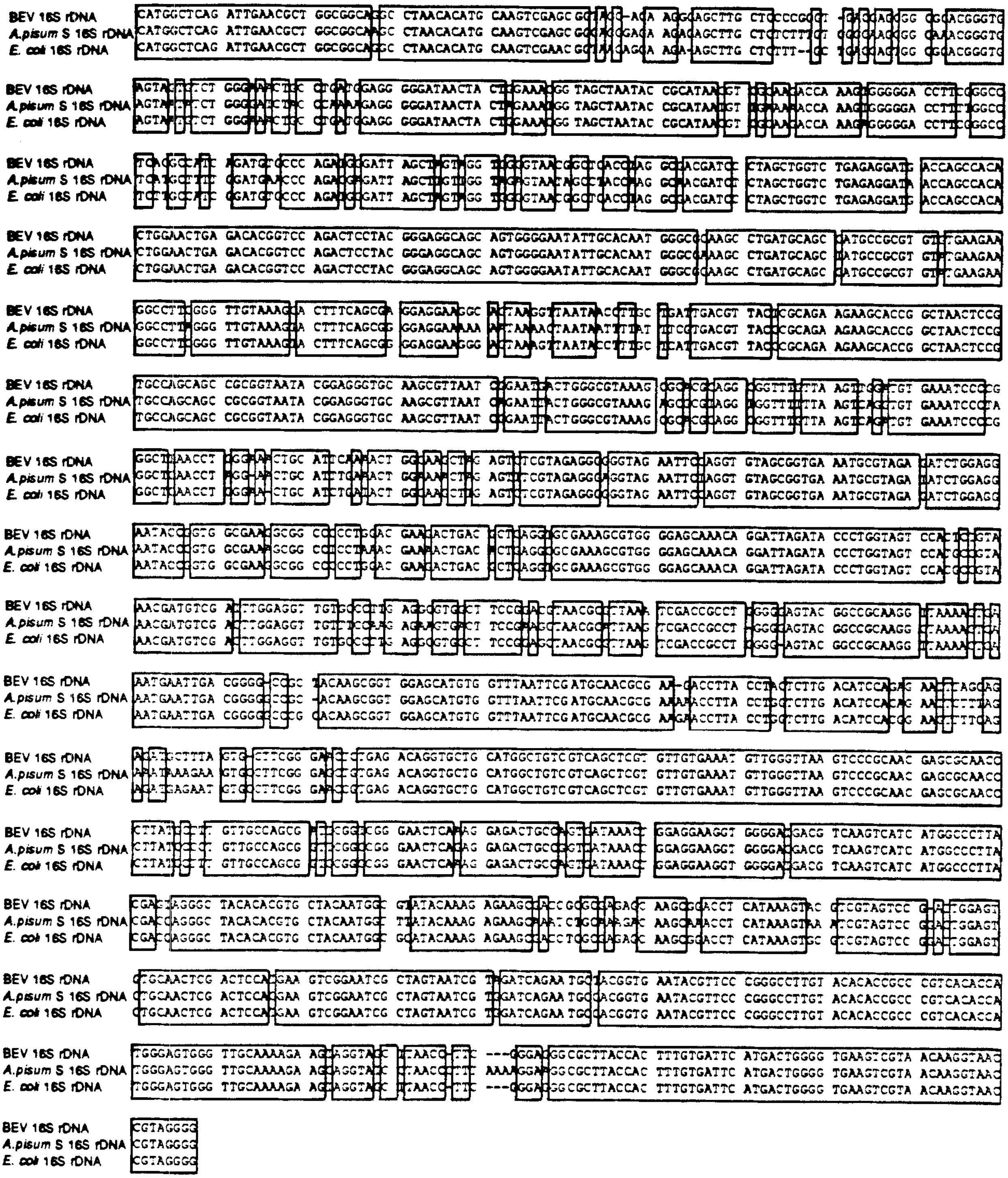

Fig. 1. Alignment of 1498 bp portion of the 16S rDNA (amplified by PCR) from BEV, a bacterial symbiote of the leafhopper *Euscelidius variegatus*, to the 16S rDNAs of the secondary symbiote of the aphid, *Acyrthosiphon pisum* (nucleotides 18-1522) and *Escherichia coli* (nucleotides 18-1516). Boxes indicate positions of nucleotide homology in all three bacteria. Each full line of sequence consists of 100 bases.

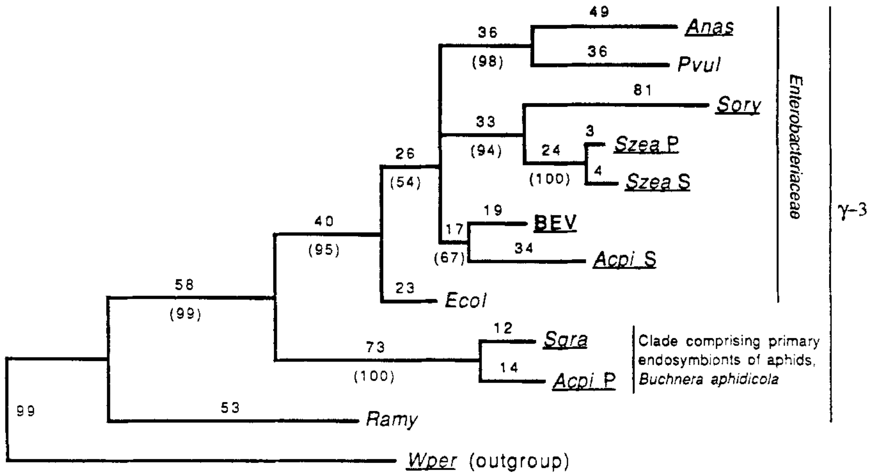

Fig. 2. Phylogenetic tree showing evolutionary affiliation of BEV within the *Enterobacteriaceae*. The tree was generated by phylogenetic analysis of 16S rDNAs on the basis of maximum parsimony by use of the consensus of 50% majority-rule of 100 bootstrap replicates. Indices in parentheses indicate bootstrap confidence levels for particular nodes; numbers not in parentheses are branch lengths. Bacteria include representatives of the γ-3 subdivision except for *Wolbachia persica*. Underlined bacteria are those which have a symbiotic relationship with various arthropods. The bacteria are abbreviated as follows: *Anas* (*Arsenophonus nasoniae*), *Pvul* (*Proteus vulgaris*), *Sory* (symbiote of *Sitophilus oryzae*), *Szea P* (primary symbiote of *Sitophilus zeamais*), *Szea S* (secondary symbiote of *Sitophilus zeamais*), *BEV* (symbiote of *Euscelidius variegatus*), *Acpi S* (secondary symbiote of *Acyrthosiphon pisum*), *Ecol* (*Escherichia coli*), *Sgra* (symbiote of *Schizaphis graminum*), *Acpi P* (primary symbiote of *Acyrthosiphon pisum*), *Ramy* (*Ruminobacter amylophilus*), and *Wper* (*Wolbachia persica*, outgroup). Tree length = 734 steps; consistency index = 0.619; f-ratio = 0.21.

phylogenetic assemblage, which includes BEV and the secondary symbiote of the pea aphid.

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